Environmental influences on neuromorphology in the non-native starling, Sturnus vulgaris

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Running head: Brain size variation in an invasive bird

Key words: starling, brain, avian hippocampus, bird, telencephalon, spatial memory, invasive species, ecology

Abstract

Cognitive traits are predicted to be under intense selection in animals moving into new environments, and may determine the success or otherwise of dispersal and invasions. In particular, spatial information related to resource distribution is an important determinant of neural development. Spatial information is predicted to vary for invasive species encountering novel environments. However, few studies have tested how cognition orneural development vary intraspecifically within an invasive species. In Australia, the non-native common starling, *Sturnus vulgaris*, inhabits a range of habitats that vary in seasonal resource availability and distribution. We aimed to identify variation in brain mass and hippocampus volume of starlings in Australia related to environmental variation across two substantially different habitat types. Specifically, we predicted variation in brain mass and hippocampal volume in relation to environmental conditions; latitude and climatic variables. To test this, brain mass and volumes of the hippocampus and two control brain regions (telencephalon and tractus septomesencephalicus) were quantified from starling brains gathered from across the species' range in SE Australia. When comparing across an environmental gradient, there was a significant interaction between sex and environment for overall brain mass, with greater sexual dimorphism in brain mass in inland populations, compared to those at the coast. There was no significant difference in hippocampal volume in relation to environmental measures (N = 17 hippocampus volume), for either sex. Whilst these data provide no evidence for intraspecific environmental drivers for changes in hippocampus volume in European starlings in Australia, they do suggest environmental factors contribute to sex differences in brain mass. This study identifies associations between brain volume of a non-native species and environment; further work in this area is required to elucidate the mechanisms driving this relationship.

Introduction

To succeed in new, challenging environments, animals must utilise complex spatial information, as well as learn and retain the location of key resources which are essential for their survival and reproduction (Sol *et al.* 2005). A range of factors dictates such cognitive abilities, including the development of the brain, along with the associated internal structures, which are associated with particular tasks (Lefebvre *et al.* 2004). At the species level brain size is commonly, although contentiously, linked to the ability to process and store complex social and spatial information (Healy & Rowe 2007). Comparative studies within birds have documented the potential impact of social groupings, sexual selection, breeding group composition or developmental mode on brain size (see, Emery 2006; Garamszegi *et al.* 2005; Iwaniuk & Arnold 2004; Willemet 2013). Together, such studies highlight the complexity of the selective forces at work causing these intriguing interspecific differences in brain size. Across species, relative brain size presumably represents the outcome of contrasting selection on cognitive capacity, whilst the increased associated metabolic demands may (Clark & Sokoloff 1999) or may not (Isler & Schaik 2006) represent a biologically meaningful constraint on brain size.

Invasive species have particular challenges, as arriving in new environments involves selection on phenotypic characteristics which promote survival, dispersal and rapid reproduction (Blackburn *et al.* 2009). It seems reasonable therefore to suppose that the evolutionary forces acting to select invasive phenotypes also affect neuromorphology. One interspecific study, comparing a large number of historic avian introduction events concluded that, at least for birds, invaders that are more successful tend to be species with relatively larger brains (Sol *et al.* 2005). Not only this, but analyses suggested that their ability to succeed was linked to their ability to innovate, suggesting that cognitive

71 mechanisms are involved in determining the success of invasions (Sol *et al.* 2005). The
72 challenges of invasions are exacerbated in highly variable environmental conditions,
73 because environmental heterogeneity is associated with a need to retain both spatial
74 and temporal information, potentially over large scales. This 'cognitive buffer hypothesis'
75 (Sol 2009) suggests that larger brains allow animals to withstand seasonal or spatial
76 variation in resource availability. Consistent with this interpretation, South American
77 parrots species inhabiting climatically more variable environments tend to have larger
78 brain sizes (Schuck-Paim *et al.* 2008). However, there are a number of problems
79 associated with such broad interspecific comparisons (Gonda *et al*. 2013). First, different
80 species inhabit different environmental conditions and so the impact of environmental
81 variables on neural development may not be comparable. Second, understanding the
82 nature of the biological impact is difficult when comparing neuromorphology at the gross
83 level of absolute brain mass, which seems unlikely to directly control any one single
84 cognitive trait.

85 The first of these problems can be tackled through intraspecific studies, assessing how
86 neuromorphology is selected across environmental gradients. For example, examining
87 variation in morphology of chickadee populations across a gradient of climate
88 predictability, Kozlovsky *et al.* (2014) showed that populations living in hasher
89 environmental conditions have larger brains. Interestingly, they also found that across
90 populations, there was a strong negative relationship between brain mass and mass of
91 the digestive tract. They interpret these data in line with the 'expensive tissue
92 hypothesis' which suggests an energetic trade-off between the development and
93 maintenance of brain and digestive tract investment. However looking across bird
94 species, there appears to be only weak support for this hypothesis (Isler & van Schaik
95 2006).

As a way of addressing the second of these problems, researchers have tried to look at key areas in the brain associated with known cognitive tasks. The role of the hippocampus has been extensively studied in food caching birds (Healy *et al.* 1994; Lee *et al.* 1998; Sherry *et al.* 1992). Despite some interesting inconsistencies (Garamszegi & Lucas 2005), it seems that both within (Pravosudov *et al.* 2006) and between species (Sherry *et al.* 1992), greater use of spatial memory is associated with a more developed hippocampus (Biegler *et al.* 2001). Hippocampal development seems to be driven by evolutionary pressures related to the value of spatial information in relation to resource availability, whether it is food, reproductive opportunity, or some other limiting resource (Mayer *et al.* 2013). Associations between relatively larger hippocampus volume and/or better performance of spatial tasks (Healy *et al.* 2005), have been criticised for making the assumption that size relates to better functional outcomes (Roth *et al.* 2010). However, recent studies have sought to relate functional outcomes with measures of neuron generation and synapse formation with promising results (Barnea & Pravosudov 2011). It has been proposed that inter-population comparisons within species, where populations differ in clearly defined ecological variables, represent a powerful way to test the ecological processes driving hippocampus evolution (Pravosudov & Clayton 2002; Pravosudov & Roth 2013).

In this study we sought to address the evolutionary pressures leading to changes in brain size and hippocampal development across the range of an invasive species. The common starling, *Sturnus vulgaris*, was introduced into Australia at several locations in the mid to late 1800's (Long 1981) and has since spread to encompass a range ofenvironments across Australia's east coast. At the landscape scale this distribution covers areas which differ in habitat suitability and also in environmental predictability. On the eastern coastline of New South Wales, Australia, starlings inhabit landscapes characterised by highly productive pastures, whilst those living inland at the range edge

encounter, arid landscapes, with a much reduced degree of agricultural development and patchily placed, unpredictable resources (Higgins *et al.* 2006). Significant variation in morphological parameters was identified in contemporary populations across environmental clines (Cardilini *et al.* 2016). Potentially, multiple processes may influence neuromophology in starlings over habitats with significant environmental variation, driven by differences in resource distribution and predictability between habitats (Shuck-Paim *et al.* 2008; Kozlovsky *et al.* 2014). Coastal populations have higher densities of starlings and are characterised by wetter conditions with the suggestion that foraging resources may be more widely distributed. In comparison, populations along the edge of the inland range are sparser, subject to drier conditions and are likely to encounter more patchily distributed food resources. To the best of our knowledge no study has ever tested the impact of environmental variables on the evolution of brain morphology within an invasive species. Consequently, the aim of this study was to determine whether brain size and hippocampus volume of starlings in south eastern Australia is significantly related to environmental variation in climatic variables.

Materials and Methods

Sample collection

Adult starlings (30 females, 38 males) were collected from 8 localities from across eastern Australia (210 km & 990 km, minimum and maximum distance between sites) between 28th April and the 2nd June 2012 (fig. 1, table I). Birds were shot with a shotgun loaded with birdshot. Global positioning system (GPS) co-ordinates were taken at the collection site of each individual. Collections were made at eight sites in New South Wales in wet productive landscapes at the centre of the starling range, and ‘inland’ at three sites on the edge of the starlings range in semi-arid landscapes of low productivity. Immediately after collection the birds were decapitated and the heads placed in individual plastic containers of formaldehyde solution (36.5-38% in H_2O; Sigma-

148 Aldrich®); care was taken to ensure that the entire head was submerged in excess
149 formaldehyde. The heads were left to fix for a minimum of one month at 4°C. Each brain
150 was then extracted from the skull and placed in a new container of formaldehyde and
151 stored at 4°C.

152 *Morphological and Environmental data*

153 Body mass (± 0.1 g) was recorded upon collection (before removing the head) and used
154 as a proxy for size. A proxy for body condition, the scaled mass index (SMI), was
155 calculated following Peig and Green (2009) referred to below as 'body condition'. SMI
156 relates individual mass and tarsus length with population values and has been shown to
157 be a good proximate measure of body condition (Peig & Green 2010). Sex was
158 determined anatomically. For samples where anatomical information was not available,
159 genetic sexing was used (Fridolfsson & Ellegren 1999); see Supplementary file 1 for
160 specific details. Wet brain mass following fixation was measured using analytical scales
161 (± 0.001 g). Environmental variables included latitude (°S), which was derived from GPS
162 co-ordinates. For each sample, location climatic variables were extracted from Bioclim
163 data sets (bio01-19) (Hijmans *et al.* 2005) and aridity data was extracted from the
164 CGIAR-CSI Global-Aridity and Global-PET Geospatial Database (Zomer *et al*. 2007;
165 Zomer *et al*. 2008). The 'raster' package in R was used to extract the climate and aridity
166 data (Hijmans 2015).

167 *Sectioning and methods*

168 A subset of twenty eight brains (7 female, 21 male; table 1; limited by funding) was cryo-
169 protected in sucrose solution in 0.1M phosphate buffer (15% followed by 30% sucrose;
170 both for 24h at 4°C). Brains were then frozen on dry ice and stored at -80°C until
171 sectioning. Brains were coronally sectioned at 60 microns, using a freezing microtome.
172 All sections were mounted on gelatinized microscope slides and then stained with

haematoxylin and eosin and serially dehydrated through ethanol and cover-slipped with DPX mountant (Sigma, St Louis, MO, USA). Slides were scanned on a Nikon COOLSCAN V ED slide scanner (LS-50ED; resolution = 4800 dpi true optic resolution, saved to 8-bit grey scale) and the resulting images were analysed using Image J (version 1.49n). There was no magnification at scanning, only during image analysis. Brain region volumes (mm^3) were calculated from the sequential areas of each region across the slides, $V = tx_i + tx_{i+1} + \ldots + tx_{i+n}$; where V is volume, t is section depth (0.06 mm) and x is the measured area of the section. The volumes (mm^3) of the left and right hemisphere of each of the following region were calculated: telencephalon, hippocampus and the tractus septomesencepahlicus (TrSM) (Stokes *et al.* 1976). The telencephalon and TrSM were included as control regions in order to compare with the hippocampus. Where a section of brain was not measured, the measurement of the proceeding section was used in its place. For the hippocampal, TrSM and telencephalon regions respectively an average of 54%, 86% and 53% of all sections were measured and used to reconstruct the volume. Hippocampus and telencephalon volumes were calculated from up to 30 sections; telencephalon volume included the hippocampus volume. To assess the repeatability of the image analysis each brain region was remeasured three times in 37 sections. The results showed that this measurement technique was reliable to ± 0.02 pixels/mm.

Statistical Analysis

A Principle Component Analysis (PCA), including all Bioclim variables and aridity, was run to help determine the environmental difference between collection localities. A PCA plot showed clear environmental differences between collection localities (Supplementary fig. 1). The first principle component (PC1), which explained 68.2% of the environmental variation, was used as a composite environmental measure for testing the effect of environmental variation on brain measurements (Supplementary table 1).

PC1 represented variation in temperature and rainfall seasonality, with negative values indicating colder, wetter environments within collection localities along the coast and positive values indicating drier, hotter environments inland. We use the terms ‘coast’ and ‘inland’ to refer to two environmentally distinct groupings of collection localities. The PCA was run in R using the ‘prcomp()’ function (R Core Team 2015).

The effects of sex, PC1 and latitude on individual mass and body condition were tested using multivariate linear regression models.

A multivariate linear regression model was used to test the influence of PC1 on individual brain mass. Bird mass was also included in the model as an independent covariate. An interaction term of sex and PC1 was included in the model to test differences in response between sexes.

There was no significant difference between the volume of right and left hemisphere measurements ($P = 0.540$), so measures were combined to create a total volume which was used as the dependent variable in linear regression models testing the relationship between brain regions and PC1. Where only one side of a region had an estimated volume, the sample was excluded from analysis. PC1 and sex were included in the models as independent variables for the hippocampus and TrSM. To test for the effect of brain size, telencephalon volume was included along with the other standard independent variables (PC1 and sex) in an alternative model. Models testing telencephalon size were also run and include the same independent variables as those included for hippocampus and TrSM models except that bird mass was included as an independent variable. We found no relationship when testing the effect of an interaction between sex and PC1 on brain region volume; we chose to exclude the interaction term from brain region models to reduce the number of independent variables being tested with small sample sizes. Results were considered significant if the *p*-value was less

than 0.05. Complete brain region section data was not available for all samples, which resulted in different sample sizes for the analysis of each brain region (Supplementary table 2).

We also tested transect for all response variables and found the same results as PC1. We were unable to separate the effects of location vs environmental variables and chose to focus on environmental variation in the discussion.

Before running the models all variables were normalised around a mean of zero and with a standard deviation of 1 to make parameter estimates comparable. All analyses were conducted in the statistical software, R, using base functions, and models run using the 'lm()' function (R Core Team, 2015). Figures were produced using the graphical package 'ggplot2' (Wickham 2009).

Results

Males were significantly heavier than females in terms of body mass (30 females, mean 75.35 g and range 66.8 g – 83.3 g, and 38 males, mean 79.76 g and range 68.8 g – 88 g; Est = 0.909, SE = 0.227, t_{64} = 4.012, P < 0.001, R^2 = 0.176). Starling body mass was not significantly related to PC1 (Est = -0.163, SE = 0.123, t_{64} = -1.328, P = 0.189) and there was no relationship between body mass and latitude (Est = 0.052, SE = 0.135, t_{64} = 0.388, P = 0.699). Body condition did not vary significantly between sexes (29 female and 35 male birds; Est = -0.032, SE = 0.2505, t_{60} = -0.128, P = 0.898, R^2 < 0.001), environment (Est = -0.158, SE = 0.137, t_{60} = -1.154, P = 0.253) or latitude (Est = -0.236, SE = 0.146, t_{60} = -1.624, P = 0.110).

Variation in relative and absolute brain mass was explained by a significant interaction between sex and PC1, with males having heavier brains than female starlings. This sexual dimorphism in brain mass was more pronounced in inland populations. Inland

males had heavier brains than coastal males, whereas inland females had lighter brains than coastal females (table II, fig. 2; brain mass by PC1within each sex; female, P 0.017, R2 = 0.159, male, P = 0.031, R^2 = 0.098). There was no relationship between brain mass and body size or latitude (table II). Brain mass did not show a relationship with body condition (Est = -0.067, SE = 0.116, t_{59} = -0.581, P = 0.564, R^2 = 0.301), when controlling for an interaction of sex and PC1.

Hippocampus volume did not show a significant relationship with PC1 or sex (table III). There was a trend for TrSM to be larger with higher values of PC1 and it was significantly larger in brains with greater telencephalon volume, but there was no relationship with sex (table III). Telencephalon showed no indication of a relationship with PC1 (table III).

Discussion

Species that can invade successfully require the ability to innovate (Diquelou *et al.* 2015) and as such, need a range of motor and cognitive skills to be able to thrive in challenging, novel environments (Griffin *et al.* 2014; Sih *et al.* 2011). The ability to respond to new environmental challenges with innovative behaviours is likely to influence survival and the chance of successful reproduction in the new range. Interspecific comparisons suggest this ability is thought to be in part determined by overall brain size (Sol *et al.* 2005), but no study to date has tested this within the range of an invasive species. In this study, we set out to test first if there was a relationship between overall brain mass and environmental conditions for the invasive European starling in Australia. Our data show that environmental conditions have an effect on sexual dimorphism in overall brain mass in this species. Second, we predicted that hippocampal volume would vary significantly in relation to environmental variation across the starling's habitat range, but our data provide no evidence for any such

pattern. This may be because selection on hippocampal volume does not occur across this spatial scale, or because there is no difference in the benefit of spatial memory between these sites. This latter possibility seems unlikely given the differences in starling distribution across the range (West 2008), which suggests greater resource density in coastal populations. Finally, it may be that the sample size of individuals measured for this study was not sufficient to be able to demonstrate any population level differences in hippocampal investment. We would argue this is unlikely, as previous work utilising similar sample sizes have been able to demonstrate interpopulation differences (Pravosudov *et al.* 2006).

Our data show (Fig II) that sexual dimorphism in overall brain mass increases when moving from the coast to inland starling populations. Such sexual dimorphism in brain mass has been previously reported (Casto & Ball 1996), but our study is the first to report the influence of environmental conditions. Interpreting these differences is challenging without accompanying behavioural or dispersal data (Healy & Rowe 2007), but there are a number of possible interpretations. Functionally, these sex differences in brain mass suggest that environmental conditions across the range in south-eastern Australia have sex-specific effects on life history traits associated with brain structure, traits such as female natal dispersal or male song production (Bernard *et al.* 1993; Ball *et al.* 1994). While several studies show that brain mass can vary between environments, it is important to note that such variation is difficult to interpret, because brain mass is a composite measure of many brain regions that may respond independently to different conditions (Healy & Rowe 2007). Mechanistically, it seems possible that a single unmeasured brain region contributed to the overall brain mass differences. In this population of Australian starling body mass varies with maximum temperature in accordance with Bergmann's rule (Cardilini *et al.* 2016), where birds are smaller in areas with hotter climatic conditions. A similar pattern (non-significant) was

detected in this dataset and may in part underlie the increased sexual dimorphism in brain size at inland sites, where residual brain mass is greater in males than in females.

Whilst we were able to demonstrate sex differences in the relationship between environmental conditions and brain mass, this study found no difference in avian hippocampus volume between populations of an invasive species that inhabit environments that differ substantially in resource distribution and predictability (Morton *et al.* 2011). Further work is required first to test the physiological impact of environmental stress on hippocampal development across populations of an invasive species (Pravosudov & Clayton 2002). Second, an integrated understanding is needed of the costs and benefits of spatial memory, in order to predict the likely selection pressures working on hippocampal development in an invasive bird, but it seems possible that population differences could be used to infer the benefit of spatial information. Future research needs to be conducted to investigate potential differences in the inland habitats that might drive differences in neuromorphology and cognition.

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441 **Table I**. Collection locality co-ordinates and the number of samples from each locality, by sex.

Collection Locality	Latitude	Longitude	Transect	No. brains (female, male)	No. sectioned (female, male)
Bega	36.618	149.871	Coastal	1, 2	1, -
Nowra	34.875	150.691	Coastal	4, 6	-, 3
Maitland	32.761	151.720	Coastal	6, 4	1, 3
Austral Eden	31.014	152.938	Coastal	6, 4	2, 1
Lismore	28.799	153.238	Coastal	5, 3	2, 3
Nyngan	31.572	147.230	Inland	3, 6	-, 6
Moree	29.736	149.846	Inland	2, 6	1, 4
Lemon Tree	27.790	151.281	Inland	3, 7	-, 1
Total				30, 38	7, 21

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443

444 **Table II**. Output of the linear regression model testing the relationship between environmental variables and starling A. relative and B. absolute

445 brain mass. For a definition of variables see text. Variables in bold indicate those with a p-value below 0.05.

Response variable	Predictor variable	Estimate	Std. Error	T-value	*p*-value
A. Brain mass	Intercept	-0.662	0.177	-3.733	<0.001
N = 68 samples	**Sex * PC1**	**0.713**	**0.218**	**3.276**	**0.002**
R^2 = 0.315	**Sex**	**1.040**	**0.243**	**4.285**	**<0.001**
DF = 64	**PC1**	**-0.407**	**0.174**	**-2.337**	**0.023**
	Mass	0.025	0.120	0.211	0.833
B. Brain mass	Intercept	-0.677	0.161	-4.205	<0.001
N = 68 samples	**Sex * PC1**	**0.719**	**0.214**	**3.356**	**<0.001**
R^2 = 0.325	**Sex**	**1.063**	**0.214**	**4.975**	**<0.001**
DF = 64	**PC1**	**-0.415**	**0.169**	**-2.466**	**0.016**

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447

448 **Table III**. Output of linear regression models testing the relationship between environmental variables and the volume of starling brain regions.

449 For a definition of variables see text. Models including and without volumetric controls are included.

Response variable	Predictor variable	Estimate	Std. Error	t-value	*p*-value
A. Hippocampus volume	Intercept	-0.015	0.211	-0.073	0.943
N = 17 samples	**Tel. Vol.**	**0.653**	**0.239**	**2.732**	**0.017**
R^2 = 0.242	PC1	-0.327	0.235	-1.394	0.187
DF = 13	Sex	-0.113	0.234	-0.484	0.636
B. Hippocampus volume	Intercept	<-0.001	0.245	-0.003	0.998
N = 18 samples	PC1	-0.136	0.266	-0.512	0.616
R^2 = -0.074	Sex	0.216	0.256	0.845	0.411
DF = 15					
A. TrSM volume	**Intercept**	**0.689**	**0.180**	**3.825**	**0.003**
N = 14 samples	**Tel. Vol**	**0.424**	**0.184**	**2.304**	**0.044**
R^2 = 0.310	PC1	-0.457	0.213	-2.139	0.058
DF = 10	Sex	0.3114	0.210	0.544	0.598
B. TrSM Volume	Intercept	-0.039	0.206	-0.188	0.852
N = 25 samples	PC1	-0.134	-0.238	-0.564	0.579
R^2 = -0.027	Sex	-0.149	0.240	-0.618	0.543
DF = 22					
A. Telencephalon volume	Intercept	-0.012	0.199	-0.159	0.954
N = 17 samples	**Brain Mass**	**0.707**	**0.259**	**2.728**	**0.173**
R^2 = 0.422	PC1	-0.032	0.240	-0.133	0.896
DF = 13	Sex	0.054	0.233	0.233	0.819

B. Telencephalon volume	Intercept	<0.001	0.231	0.000	1.000
N = 17 samples	Mass	-0.018	0.411	-0.045	0.965
R^2 = 0.092	PC1	0.286	0.306	0.936	0.366
DF = 13	Sex	0.385	0.344	1.120	0.283

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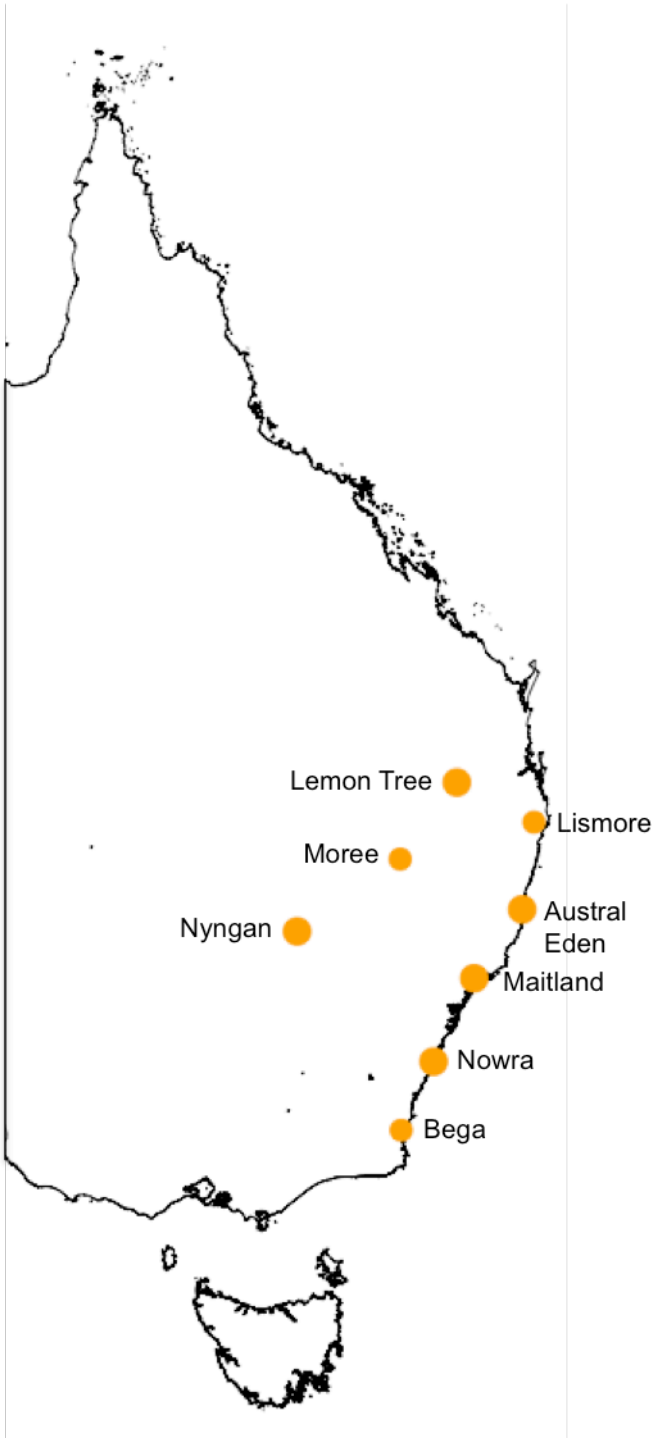


Fig 1. Map of eastern Australia where the circles represent collection localities and the size of the circles represents the number of samples collected. The number of samples ranged from 8 to 10.

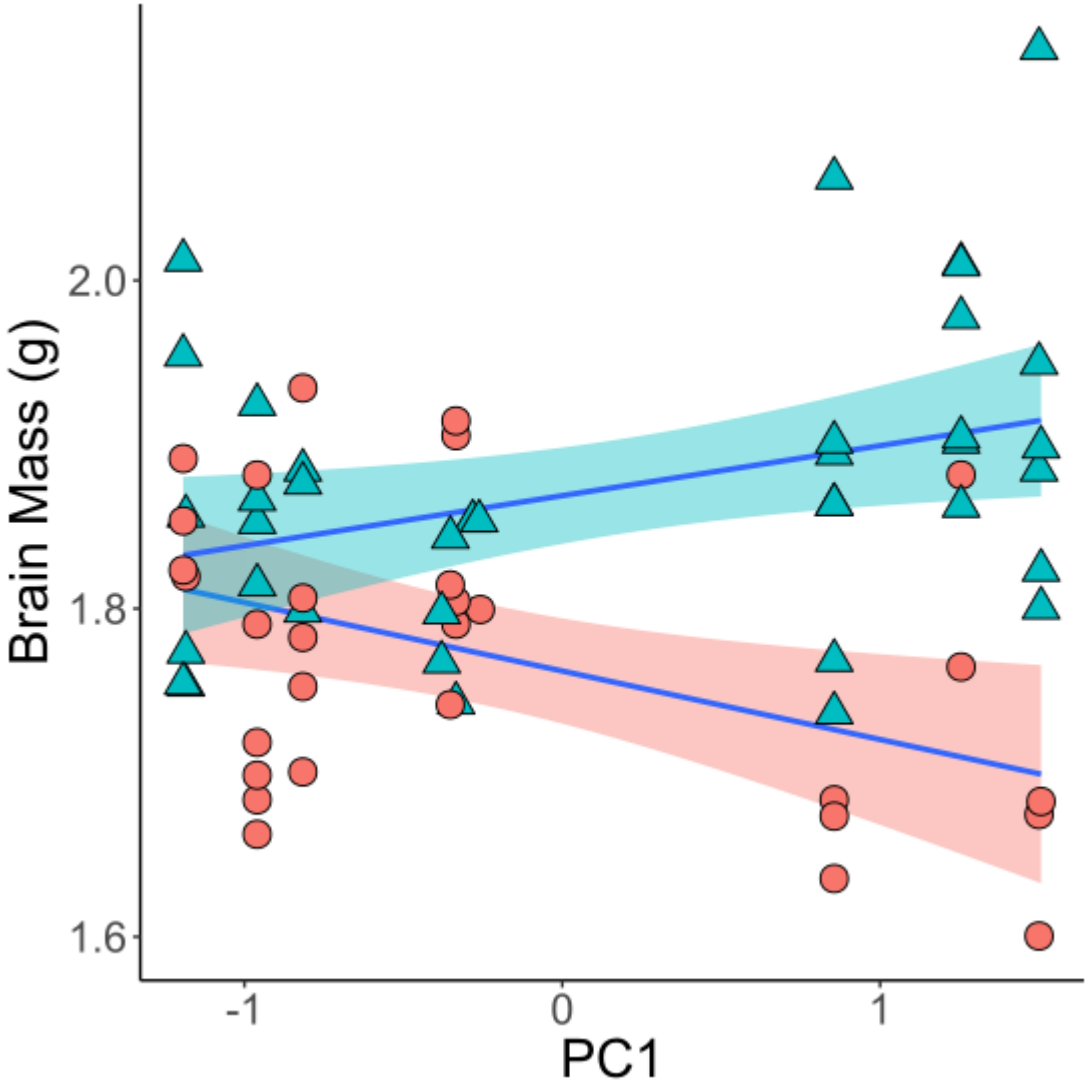
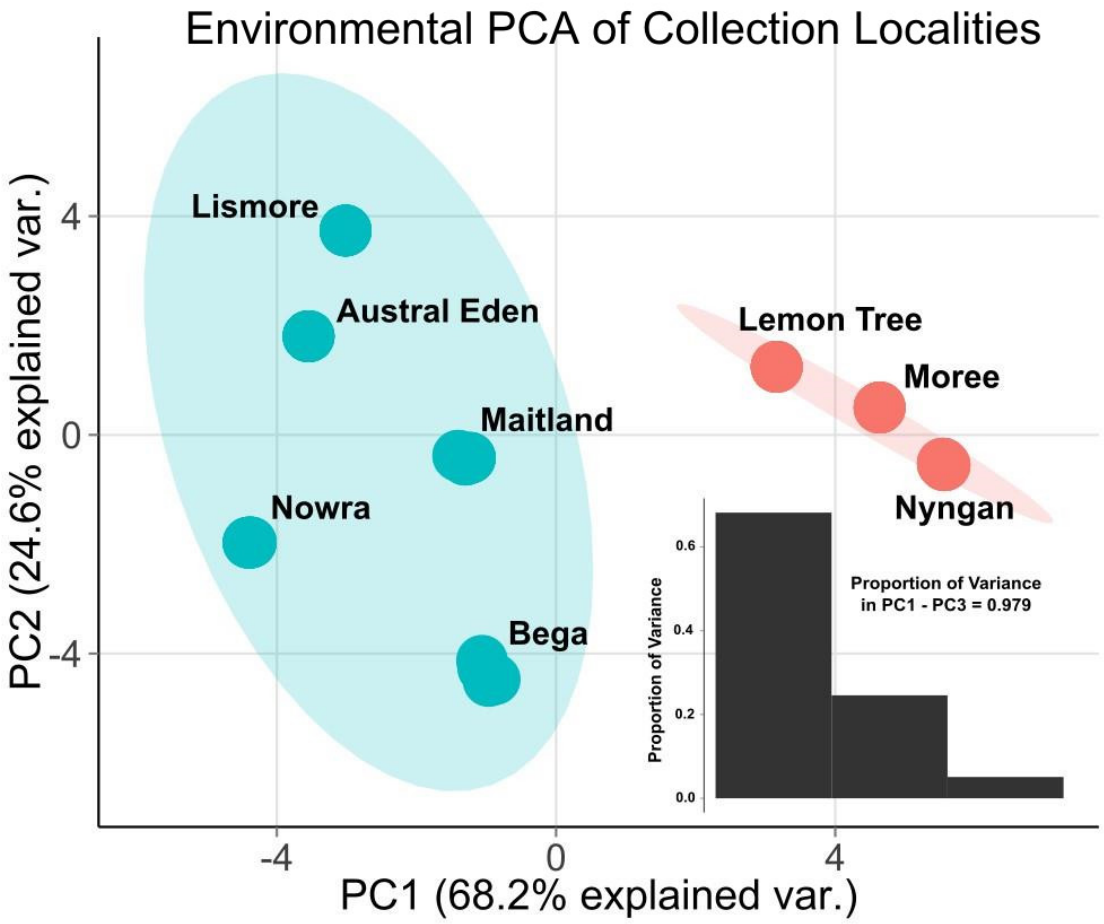


Fig 2. Lines represent the predicted values from a multivariate linear regression model testing the relationship between brain mass as the dependent variable, sex*pc1 as an interaction term, and bird mass as independent variables. Circles represent females and triangles represent males. The points represent the real brain mass values.



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464 **Supplementary Fig 1**. PCA plot of collection localities in environmental space. PC1 and
465 PC2 were derived from 20 environmental variables. The colour of a point indicates the
466 environmental cluster it falls within, and the coloured ellipse represents the 95%
467 confidence interval for each cluster. A histogram of eigenvalues indicates the proportion
468 of variance described by PC1 – PC3.

469

PC Loadings of Environmental PCA Variables

Supplementary Table 1. Statistics and PC loadings from a PCA analysis of 20 environmental variables.

Variable	PC1	PC2	PC3
Aridity	-0.268	-0.029	0.081
Bio1 (Annual Mean Temperature)	0.072	0.418	0.249
Bio2 (Mean Diurnal Range)	0.246	0.162	-0.171
Bio3 (Isothermality)	-0.128	0.247	-0.662
Bio4 (Temperature Seasonality)	0.265	0.052	0.130
Bio5 (Max Temperature of Warmest Month)	0.240	0.192	0.149
Bio6 (Min Temperature of Coldest Month)	-0.253	0.086	0.282
Bio7 (Temperature Annual Range	0.263	0.100	-0.003
Bio8 (Mean Temperature of Wettest Quarter)	0.203	0.272	0.224
Bio9 (Mean Temperature of Driest Quarter)	-0.156	0.341	0.102
Bio10 (Mean Temperature of Warmest Quarter)	0.205	0.265	0.271
Bio11 (Mean Temperature of Coldest Quarter)	-0.179	0.329	0.144
Bio12 (Annual Precipitation)	-0.264	0.089	0.039
Bio13 (Precipitation of Wettest Month)	-0.242	0.183	-0.030
Bio14 (Precipitation of Driest Month)	-0.236	-0.180	0.125
Bio15 (Precipitation Seasonality	-0.063	0.412	-0.327

Bio16 (Precipitation of Wettest Quarter)	-0.245	0.183	-0.015
Bio17 (Precipitation of Driest Quarter)	-0.257	-0.076	0.161
Bio18 (Precipitation of Warmest Quarter)	-0.247	0.164	-0.022
Bio19 (Precipitation of Coldest Quarter)	-0.263	-0.036	0.196
Standard Deviation	3.693	2.217	1.009
Proportion of Variance	0.682	0.246	0.051
Cumulative Proportion	0.682	0.928	0.979

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Brain Region Section Analysis Sample Sizes

Supplementary Table 2. Number of samples used in models for each brain region section analysis (see Table III in paper).

Collection Locality	A. Hippo. Rel. (female, male)	B. Hippo. Abs. (female, male)	A. TrSM Rel. (female, male)	B. TrSM Abs (female, male)	A & B. Telen (female, male)
Bega	-, -	1, -	-, -	1, -	-, -
Nowra	-, 3	-, 3	-, 1	-, 1	-, 3
Maitland	3, -	3, -	3, -	3, 1	3, -
Austral Eden	1, -	1, -	1, -	2, 1	1, -
Lismore	2, 2	2, 2	2, 1	2, 1	2, 2
Nyngan	-, 2	-, 2	-, 2	-, 6	-, 2
Moree	1, 2	1, 2	1, 2	1, 5	1, 2
Lemon Tree	-, 1	-, 1	-, 1	-, 1	-, 1
Total	7, 10	8, 10	7, 7	9, 16	7, 10

Genetic sexing

DNA was extracted from muscle samples using a Gentra PureGene Tissue Kit following the manufacturer's instructions. Samples were amplified for the CHD1 gene as recommended for non-ratite birds (Fridolfsson and Ellegren 1999). The P2/P8 primers were used to amplify the CDH1 gene (Fridolfsson and Ellegren 1999). Polymerase Chain Reaction (PCR) was conducted in a total volume of 10 µl containing ~10 ng of genomic DNA, 0.3 µM of each forward and reverse primer, 0.05 U Hot Start Taq (Qiagen), 150 µM each dNTP, 1·PCR buffer (Qiagen) consisting of Tris–HCl, KCl, and (NH4)2SO4, with a final concentration of 1.5 mM $MgCl_2$. The following PCR amplification conditions were used: 30 cycles with 95°C for 30 s, 55°C annealing temperature for 30 s, and 72 °C for 30 s. An initial prolonged denaturation step (95 °C for 15 min) was included before the first cycle, and the last cycle was followed by an 8 min. extension. The amplified products were separated by electrophoresis on a 1% agarose gel and run through a 0.5·TBE buffer, at 120 volts for 45 mins, and visualised using GelRed™ (Biotium, Inc.) staining. Females are described by two distinct bands, while males are described by a single band of a different size. There was no contamination of the negative control and the positive controls were confirmed.

Reference

Fridolfsson A-K, Ellegren H (1999) A simple and universal method for molecular sexing of non-ratite birds. Journal of Avian Biology 30:116-121